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# Imatinib restores VASP activity and its interaction with Zyxin in BCR–ABL leukemic cells

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ABSTRACT

Vasodilator-stimulated phosphoprotein (VASP) and Zyxin are interacting proteins involved in cellular adhesion and motility. PKA phosphorylates VASP at serine 157, regulating VASP cellular functions. VASP interacts with ABL and is a substrate of the BCR–ABL oncoprotein. The presence of BCR–ABL protein drives oncogenesis in patients with chronic myeloid leukemia (CML) due to a constitutive activation of tyrosine kinase activity. However, the function of VASP and Zyxin in BCR–ABL pathway and the role of VASP in CML cells remain unknown. *In vitro* experiments using K562 cells showed the involvement of VASP in BCR–ABL signaling. VASP and Zyxin inhibition decreased the expression of anti-apoptotic proteins, BCL2 and BCL-XL. Imatinib induced an increase in phosphorylation at Ser157 of VASP and decreased VASP and BCR–ABL interaction. VASP did not interact with Zyxin in K562 cells; however, after Imatinib treatment, this interaction was restored. Corroborating our data, we demonstrated the absence of phosphorylation at Ser157 in VASP in the bone marrow of CML patients, in contrast to healthy donors. Phosphorylation of VASP on Ser157 was restored in Imatinib responsive patients though not in the resistant patients. Therefore, we herein identified a possible role of VASP in CML pathogenesis, through the regulation of BCR–ABL effector proteins or the absence of phosphorylation at Ser157 in VASP.



## 1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm of the hematopoietic stem cell, characterized by the presence of the fusion protein breakpoint cluster region-abelson (BCR–ABL), an oncoprotein with a constitutively tyrosine kinase activity. Significant efforts are being made to understand the molecular mechanisms of BCR–ABL signaling pathway [1]. Imatinib mesylate (Gleevec/Glivec, STI571) is a specific BCR–ABL tyrosine kinase inhibitor that can lead towards the elimination of the clone of BCR–ABL positive cells [2]; however there are patients that fail to respond to this treatment. Thus, the combination of tyrosine kinase inhibitors and other targets has been intensified in an attempt to induce apoptosis of resistant CML cells, including the strategies targeting BCL-2 protein family [3–6]. The cross-talk between the aberrant BCR–ABL signaling and BCL2 protein family has been described and has generated new therapeutic opportunities [7].

Several mechanisms influence disease evolution leading to uncontrolled cell proliferation, resistance to apoptotic and altered migration of CML cells. BCR–ABL oncoprotein induces multiple abnormalities of cytoskeletal function, resulting in an increased mobility and a reduced adhesion of leukemic cells to bone marrow stroma [8,9]. Dysregulation of actin polymerization and the depolymerization process are often associated with tumor development [10–12].

Cytoskeleton protein activity and the actin reorganization are essential to a variety of cellular processes including cell motility, cell division, cellular differentiation, and establishment and maintenance of cell adhesion [13,14]. VASP (vasodilator-stimulated phosphoprotein) and Zyxin are two important actin regulatory proteins; that act as a protein complex involved in driving actin dynamics in the context of cell division and the formation and maintenance of cell junctions [15,16]. Zyxin directs actin assembly by recruiting VASP to specific sites of adhesion [17,18].

VASP is a member of the Ena/VASP protein family. These family members share a conserved structure consisting of an N-terminal homology EVH1 domain, a central poly-proline region (PPR) and a C-terminal EVH2 domain [19]. The C-terminal of Zyxin has an N-terminal proline-rich domain and three LIM domains. The proline-rich domain may interact with SH3 domains of proteins involved in signal transduction pathways while the LIM domains are probably involved in protein-protein binding [19]. VASP interacts with Zyxin through the proline-rich region or through the LIM domain, though

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only the interaction with the LIM region is dependent on VASP's phosphorylation state [20]. VASP is a substrate of cAMP-dependent and cGMP-dependent protein kinase and phosphorylation at Ser157 in VASP regulates its interaction with actin [21]. In addition, Zyxin phosphorylation has been proposed to modulate its head–tail interaction to alter protein binding and thus cell–cell adhesion [16,22].

Altered expression of VASP and its partner Zyxin has been previously described in a variety of epithelial tumors [23–26]; however little is known regarding their involvement in hematopoietic tumors, particularly CML. VASP homologues in Drosophila were identified as substrates of the tyrosine kinase ABL [27]. In mammals, the physical interaction of VASP with ABL and its tyrosine phosphorylation were both demonstrated [28]. However, the connection of VASP and Zyxin to BCR–ABL signaling pathway in the context of CML remains unclear. In addition, the phosphorylation of VASP on Ser157 in CML leukemic cells and how this phosphorylation is affected by Imatinib treatment are not yet known. Herein we described VASP and Zyxin interaction in K562 cells before and after Imatinib treatment and phosphorylation of VASP on Ser157 status in K562 cells and in CML patient bone marrow cells. We characterized VASP and Zyxin participation and action in downstream effectors of BCR–ABL signaling.

## 2. Materials and methods

### 2.1. Bone marrow samples

Bone marrow samples were collected from 5 individuals: 1 healthy donor, 1 patient with a diagnosis of chronic myelogenous leukemia (CML), 1 responsive patient in major molecular remission treated with tyrosine kinase inhibitors and 2 patients resistant to treatment. Samples were collected between 2011 and 2012 after informed written consent and the study was approved by the Ethics Committee of the University of Campinas. Total cells from bone marrow samples were obtained after removing erythrocytes by hemolysis.

### 2.2. Cell culture and reagent chemicals

K562 cells, which are known as a representative human CML cell line, were obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal bovine serum (FBS) and glutamine with penicillin/streptomycin and amphotericin B, and maintained at 37 °C, 5% $CO_2$. Imatinib mesylate was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) and prepared as a 50 mM stock solution in dimethyl sulfoxide ($Me_2SO_4$; DMSO) and used at final concentrations of 0.1, 0.5 or 1 μM, as indicated.

### 2.3. Lentiviral vectors

K562 cells were transduced with lentivirus-mediated shRNA nonspecific control (sc-108080) or lentivirus-mediated shRNA targeting VASP (shVASP; sc-29516-V) and Zyxin (shZyxin; sc-36370-V) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, $2 \times 10^5$ cells were transduced with lentiviral by spinoculation at multiplicity of infection equal to 3 and selected by puromycin (1.75 μg/mL).

### 2.4. RNA extraction and Reverse Transcription

Total RNA was extracted using the Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Single stranded cDNA was synthesized from 2 μg of the total RNA preparation using SuperScript® III Reverse Transcriptase kit (Invitrogen Life Technologies).

### 2.5. Quantitative polymerase chain reaction

Quantitative PCR (qPCR) was performed using ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) with specific primers for *VASP*, *Zyxin* and *HPRT*. Primer sequences and concentrations are described in Supplementary Table 1. The relative gene expression was calculated using the equation, $2^{-\Delta\Delta CT}$ [29].

### 2.6. Immunoblotting

Pelleted cells were resuspended in RIPA buffer, incubated for 45 min at 4 °C and centrifuged for 30 min at 12,000 rpm at 4 °C. The same amount of protein was loaded on SDS-PAGE and blots were probed with the indicated antibodies and ECL Western Blot Analysis System (Amersham Pharmacia Biotech, UK). Antibodies against ABL (sc-23), p-ERK (sc-7383), P70S6K (sc-8418), CRKL (sc-319), BAX (sc-20067), Actin (sc-1616), p-P70S6K (sc-7984), BCL-XL (sc-8392), BCL2 (sc-492), p-BAD (sc-7999), BAD (sc-943), p-STAT 5 (C11C5), STAT 5 (sc-835), FAK (sc-558), BAK (sc-G23) and VASP (sc-1853) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-CRKL (3181), p-p130 CAS (4011), p130 CAS (12015), p-VASP (3111), and BIM (2819) were from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA) and Anti-ERK1/2 (13-6200) was from Zymed (Invitrogen, Carlsbad, CA, USA). Antibodies against p-JNK (44690G) and JNK (446826) were from Invitrogen and antibody against p-FAK Y397 (ab4803), p-FAK Y576/577 (ab76244), p-FAK Y861 (ab38458), p-FAK Y925 (ab38512), p-Zyxin (ab78910) and Zyxin (ab58210) was from Abcam (Cambridge, MA, USA). The membranes were stripped and reprobed with antibodies for detection of the respective total proteins and of Actin as control of loading.

### 2.7. Immunoprecipitation

K562 cell lysates were prepared in RIPA buffer containing protease inhibitors, as previously described [30]. Briefly, 500 μg of total K562 cell extracts was incubated overnight with 20 μL anti-VASP antibody (Santa Cruz Biotechnology) or with normal goat immunoglobulin (IgG) as a negative control. The immune complexes were precipitated with protein-G-sepharose 50% slurry (GE), washed in RIPA buffer to remove unspecific proteins, and then analyzed by western-blotting with the antibodies of interest.

### 2.8. Confocal immunofluorescence microscopy

Confocal imaging was carried out using primary antibodies against VASP or Zyxin (diluted 1:200), as previously described [31]. Actin was stained by Phalloidin (1:1000; Invitrogen). Imaging was performed using a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) with a 63× optical zoom.

### 2.9. Methylthiazoletetrazolium (MTT) assay

Cell proliferation/viability was measured by MTT assay as previously described [32]. Cells were serum-starved in 0.5% FBS for 12 h. A total of $5 \times 10^4$ cells per well were then plated in a 96-well plate in RPMI 10% FBS. In brief, 10 μL of a 5 mg/mL solution of MTT was added to the wells and incubated at 37 °C for 4 h. The reaction was stopped by using 100 μL of 0.1 N HCl in anhydrous isopropanol. Cell proliferation/viability was evaluated by measuring the absorbance at 570 nm, using an automated plate reader. All conditions were tested in six replicates.

### 2.10. Colony formation assay

Colony formation was carried out in semisolid methyl cellulose medium. $1 \times 10^3$ cell/mL was seeded in MethoCult 4230 (StemCell

Technologies Inc.; Vancouver, BC, Canada). Colonies were detected after 8 days of culture by adding 1 mg/mL of MTT reagent and scored by Image J quantification software (U.S. National Institutes of Health).

### 2.11. Apoptosis assays

Cells were washed twice with ice cold PBS and resuspended in binding buffer containing 1 μg/mL PI and 1 μg/mL APC labeled Annexin-V.

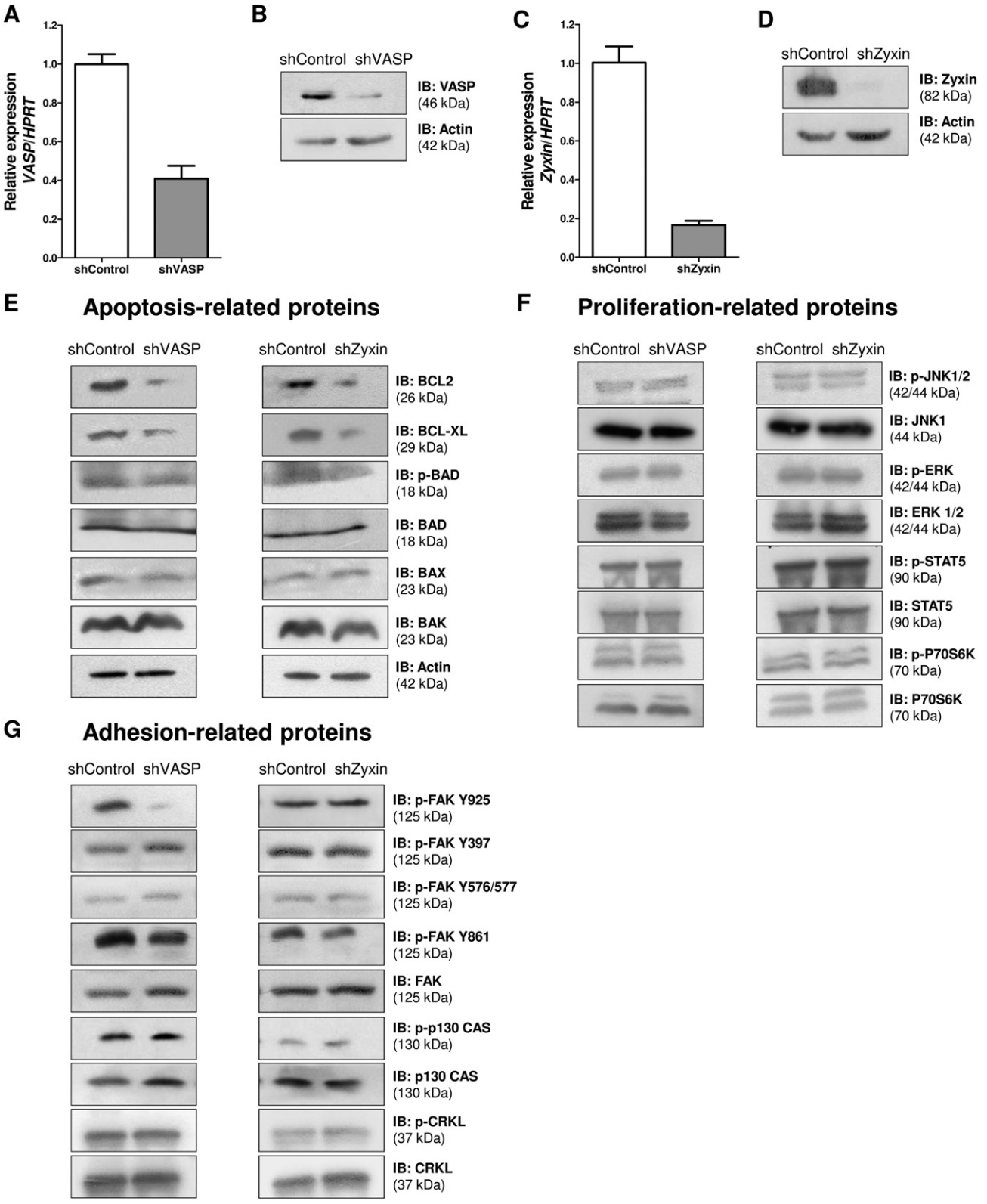


**Fig. 1.** VASP and Zyxin modulate apoptosis-related proteins. (A–D) Evaluation of VASP and Zyxin silencing in K562 cells transduced with lentivirus-mediated shRNA control and lentivirus mediated shRNA targeting VASP and Zyxin by quantitative RT-PCR analysis (A, C) and Western blotting analysis (B, D), respectively. (E–G) Western blotting analysis was used for quantification of protein expression and activity. Respective total protein or Actin was used as a control to ensuring equal sample loading; the antibodies used for immunoblotting (IB) are indicated.

All specimens were analyzed on a FACSCalibur after incubation for 15 minutes at room temperature in a light-protected area. Ten thousand events were acquired for each sample.

### 2.12. Statistical analysis

Statistical analyses were performed using GraphPad Instat 5 (GraphPad Software, Inc., San. Diego, CA, USA). For comparisons, an appropriate Student's *t*-test or analysis two-way ANOVA test and Bonferroni post-test were used. *P*-value $<0.05$ was considered as statistically significant.

## 3. Results

### 3.1. VASP participates in BCR–ABL pathway modulating effector proteins as BCL2, BCL-XL and FAK activity

In an attempt to understand the cellular effects of the interaction between VASP and BCR–ABL, we silenced VASP and Zyxin in K562 cells and analyzed the activity effector proteins in the BCR–ABL signaling pathway. The analysis showed a significant reduction by qPCR, 60% for VASP and 75% for Zyxin (Fig. 1A, 1C), as well as by western blot (81% for VASP and 99% for Zyxin) (Fig. 1B, 1D).

The silencing of VASP and Zyxin in BCR–ABL positive cells allowed us to analyze their effects upon functional studies. Briefly, we divided BCR–ABL pathway effectors into three categories: those involved in apoptosis (BCL2, BCL-XL, BAD, BAX and BAK), proliferation (JNK1/2, ERK1/2, STAT5 and P70S6K), and adhesion (CRKL, p130 CAS and FAK). The expression of anti-apoptotic proteins BCL2 and BCL-XL was reduced in VASP and Zyxin K562 depleted cells (Fig. 1E). However, there was no change in the phosphorylation or expression of pro-apoptotic proteins BAD, BAX and BAK in K562 depleted of VASP or Zyxin as compared to control cells (Fig. 1E). The activity of proliferation-related proteins was not modulated by VASP and Zyxin silencing (Fig. 1F).

Concerning adhesion proteins, VASP depletion resulted in a significant reduction in phosphorylated FAK at Tyr925, whereas Zyxin depletion did not affect activation of FAK (Fig. 1G). Activity of adhesion related proteins p130CAS and CRKL was similar in controls and VASP- or Zyxin-silenced cells.

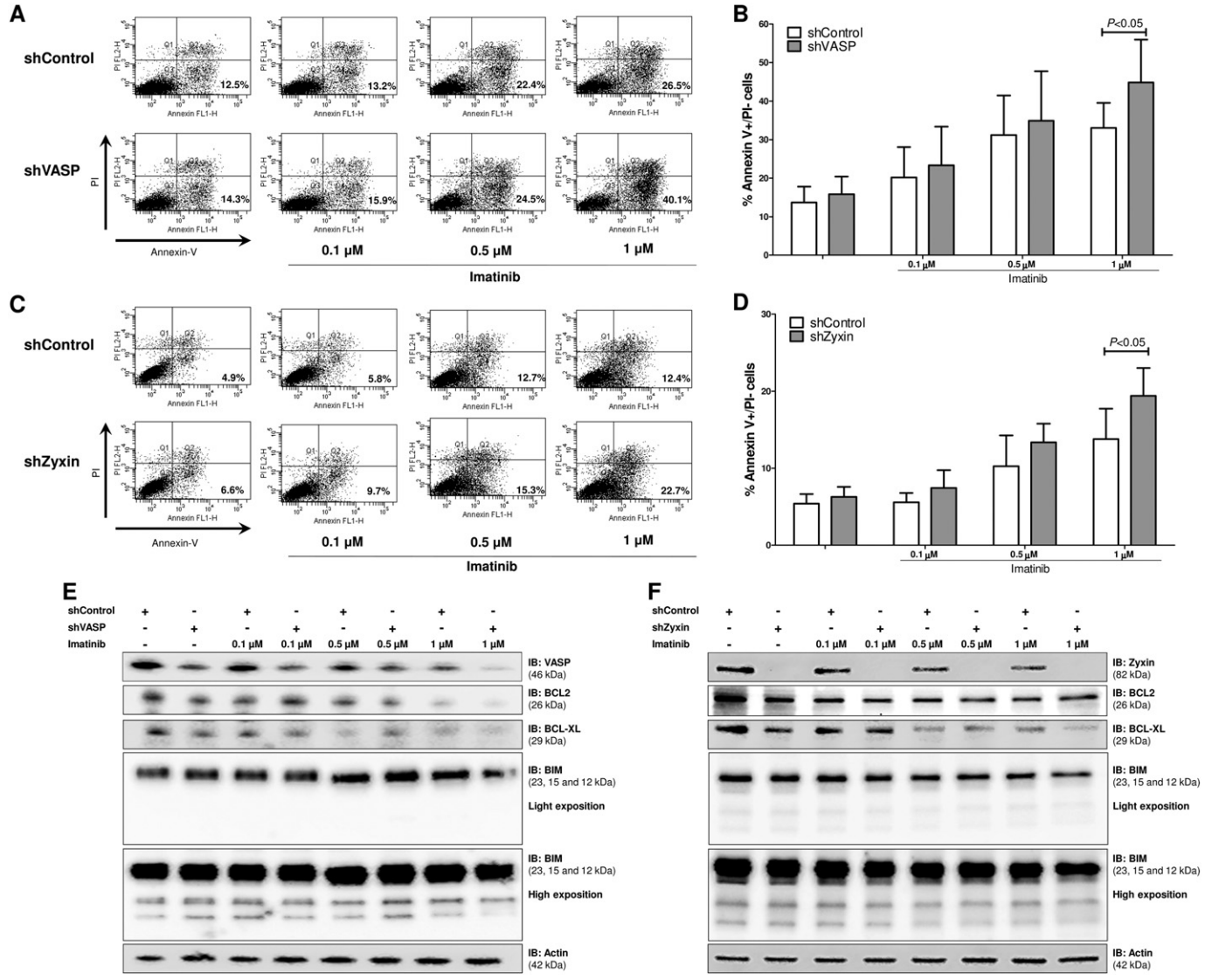


**Fig. 2.** Knockdown of VASP and Zyxin increases Imatinib-induced apoptosis. (A) and (C) Apoptosis was detected by flow cytometry in shControl, shVASP and shZyxin cells, respectively, not treated or treated with imatinib (0.1, 0.5 and 1 μM for 48 h) using Annexin-V and PI staining method. The lower right quadrant indicates the percentage of apoptotic (Annexin-V+/PI−) population. (B) and (D) Dot plots are representative of one experiments and bars graphs indicate the mean ± SD of at least three independent experiments. The *P* values are indicated in the figure; two-way ANOVA test and Bonferroni post-test. Western blotting analysis of total extracts from shControl and shVASP (E) or shZyxin (F) cells was used for quantification of protein expression and activity. Respective total protein or Actin was used as a control to ensuring equal sample loading; the antibodies used for immunoblotting (IB) are indicated.

### 3.2. VASP and Zyxin silencing increases the sensibility of K562 cells to Imatinib treatment

To evaluate the effect of VASP and Zyxin on apoptosis, cell growth and clonogenicity, K562 cells depleted of either VASP or Zyxin, as well as control cells, were treated with 0.1, 0.5 or 1 μM Imatinib and the dose–response effect of the drug was observed. VASP- and Zyxin-silenced cells had higher rates of apoptosis when treated with 1 μM Imatinib as compared to control cells ($P < 0.05$; Fig. 2A–D). These findings were in agreement with our data that suggested a higher sensibility to apoptosis, as observed by downregulation of anti-apoptotic proteins BCL2 and BCL-XL in VASP- and Zyxin-depleted cells. Of note, Imatinib treatment impacted on the BCL2 and BCL-XL expression, and the lowest levels were observed in VASP- and Zyxin-depleted cells treated with 1 μM of Imatinib (Fig. 2E–F). BIM expression was not modulated by VASP or Zyxin depletion (Fig. 2E–F).

The role of VASP and Zyxin in cell growth/viability was analyzed in an MTT assay. There was no difference in cell growth in VASP-depleted cells (Fig. 3A). However, Zyxin-depleted cells treated with 0.5 and 1 μM of Imatinib showed a significant reduction in cell viability ($P < 0.01$; Fig. 3B), which may be a consequence of the increased apoptosis. There were no significant changes in the number of colonies in shVASP and shZyxin cells compared to shControl cells (Fig. 3C and D).

### 3.3. Imatinib treatment restores VASP and Zyxin interaction in CML K562 cells

CML progression is characterized by altered adhesion and increased mobility of premature leukemic cells released from the bone marrow and this process could compromise the association between the cytoskeletal and adhesion proteins of the extracellular matrix [8,10]. The interaction of VASP and Zyxin is well characterized in epithelial tissue; however their interaction in CML is unknown. By immunoprecipitated and western blotting, we demonstrated that VASP and Zyxin do not interact in K562 cells; however, after treatment with Imatinib (1 μM), VASP and Zyxin interacted, indicating that the association of VASP and Zyxin is impaired by BCR–ABL activity (Fig. 4A). Confocal analysis did not reveal differences in Actin remodeling in cells treated with Imatinib (1 μM) for 12 h compared to control cells. However, analyses performed here are limited by the compact cytoplasm in this cell line (Supplementary Fig. 1).

### 3.4. Imatinib treatment increases VASP phosphorylation on Ser157 and reduces VASP interaction with BCR–ABL

Serine 157 is an important site of phosphorylation on VASP that regulates adhesive functions and its interactions with other proteins [21]. Here we attempted to analyze phosphorylation at Ser-157 in VASP of BCR–ABL positive cells and Imatinib effect on this phosphorylation. K562 cells were treated with Imatinib (1 μM) for 3, 6, 9 and 12 h and an increase in phosphorylation at ser157 of VASP was observed after 3 hours of treatment (Fig. 4B).

Since VASP interacts with ABL and VASP is tyrosine phosphorylated by BCR–ABL [28], we sought to study the interaction of VASP and BCR–ABL in K562 cells and the effect of Imatinib on this interaction. Imatinib treatment was performed as previously described by our group [33]. Lysates of K562 cells treated with 1 μM Imatinib for 3, 6, 9 and 12 h, as well as untreated controls, were subjected to immunoprecipitation with anti-VASP antibodies. Western blotting demonstrated the interaction between VASP and BCR–ABL; this interaction was reduced during Imatinib treatment (Fig. 4C). Taken together these results suggest that phosphorylation of VASP at serine 157 or BCR–ABL activity may modulate the association between these proteins.

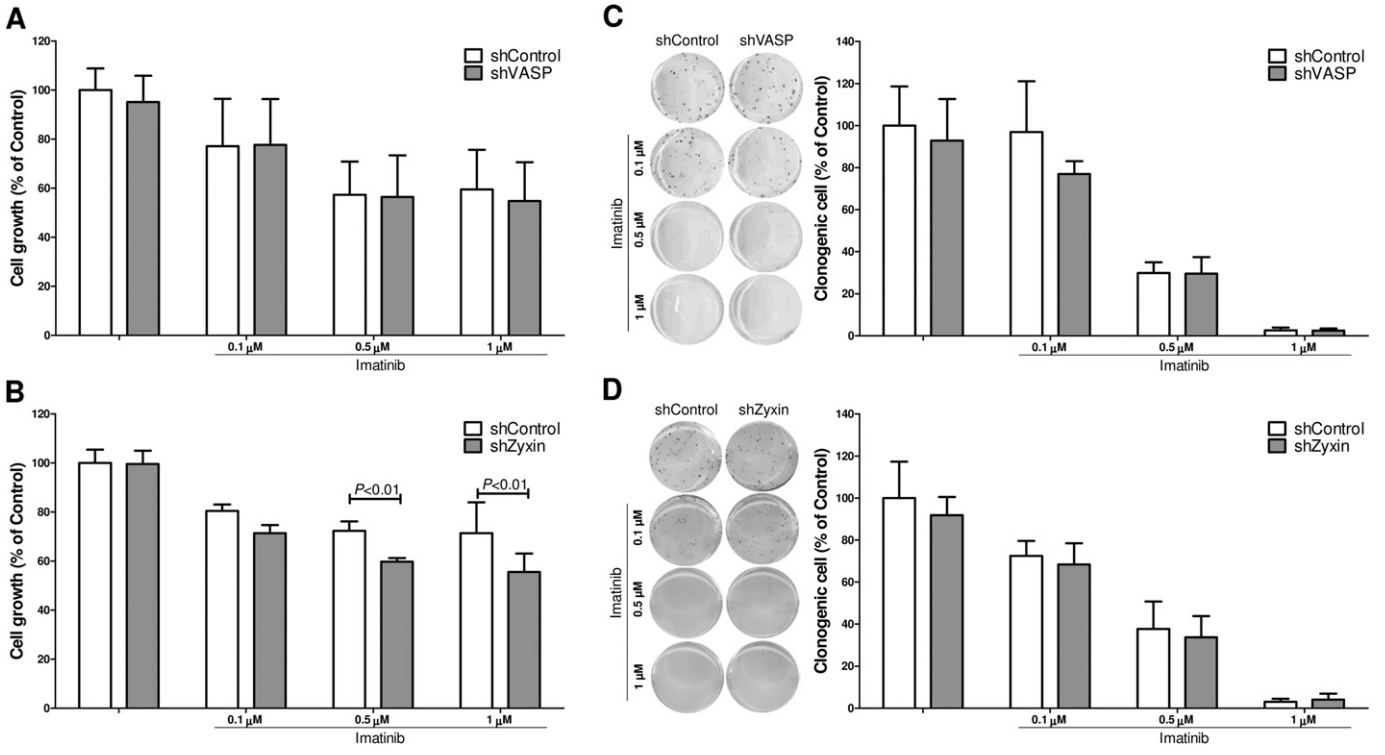


**Fig. 3.** VASP and Zyxin silencing does not modulate proliferation and clonogenicity. (A) and (B) Cell proliferation/viability was determined by MTT assay of incubation of shControl or shVASP or shZyxin K562 cells, respectively, treated or not with imatinib mesylate (0.1; 0.5 or 1 μM) after 48 h and normalized by untreated shControl cells. Values are expressed in percentage, and normalized to the shControl value set as 100%. Results are shown as mean ± SD of at least three independent experiments. The *P* values are indicated in the figure; two-way ANOVA test and Bonferroni post-test (C) and (D) shControl or shVASP or shZyxin cells were cultured in methyl cellulose and colonies were detected by MTT after 8 days of incubation with or without treated imatinib mesylate (0.1; 0.5 or 1 μM) and were normalized by untreated shControl cells. Values are expressed in percentage, and normalized to the shControl value set as 100%. Colony images are representative of one experiment and the bar graphs are shown as mean ± SD of at least three independent experiments.

### 3.5. Phosphorylation of VASP on Ser157 is absent in CML bone marrow cells

Since altered actin dynamics may be involved in tumorigenesis and VASP interaction with actin is regulated by phosphorylation in ser157, we investigated phosphorylation at Ser157 in VASP status in bone marrow cells from 1 healthy donor control and 4 CML patients (1 at diagnosis, 1 in molecular remission and 2 Imatinib resistant patients). Healthy donor cells showed phosphorylation at serine 157 of VASP, in contrast to CML cells which did not express phosphorylation at Ser157 in VASP. After Imatinib treatment, VASP phosphorylation at ser157 was restored in CML bone marrow cells; however this was not observed in Imatinib resistant patients (Fig. 4D).

## 4. Discussion

ABL protein plays a key role regulating many cellular processes, including cytoskeletal function and adhesion [34]. The constitutively active BCR–ABL fusion protein is observed in CML, where it influences the cytoskeleton and affects adhesion of CML cells to bone marrow stroma [35]. The connection between the oncogenic activity of BCR–ABL and its role in CML is unclear. Here we studied the role of VASP and its partner Zyxin in CML cells and in BCR–ABL pathway.

We studied the phosphorylation at Ser-157 in VASP in CML bone marrow cells and in K562 cells, a critical phosphorylation site for VASP activity regulation. PKA phosphorylation is known to disrupt the VASP–ABL interaction, blocking VASP binding to the SH3 domain of ABL [21,36]. Inhibition of PKA activity prevents VASP phosphorylation on serine 157 and promotes the association of VASP–ABL [37]. Those findings suggest that the absence of phosphorylation at Ser157 in VASP in CML patients and in K562 cells may promote the interaction between VASP and BCR–ABL in leukemic cells.

We demonstrated here that there is no phosphorylation of VASP at ser157 in CML bone marrow cells. After Imatinib treatment, remission patients restored phosphorylation of VASP on Ser157 expression and CML patients resistant to Imatinib did not present phosphorylation at Ser157 in VASP in their bone marrow cells. Consistent with this

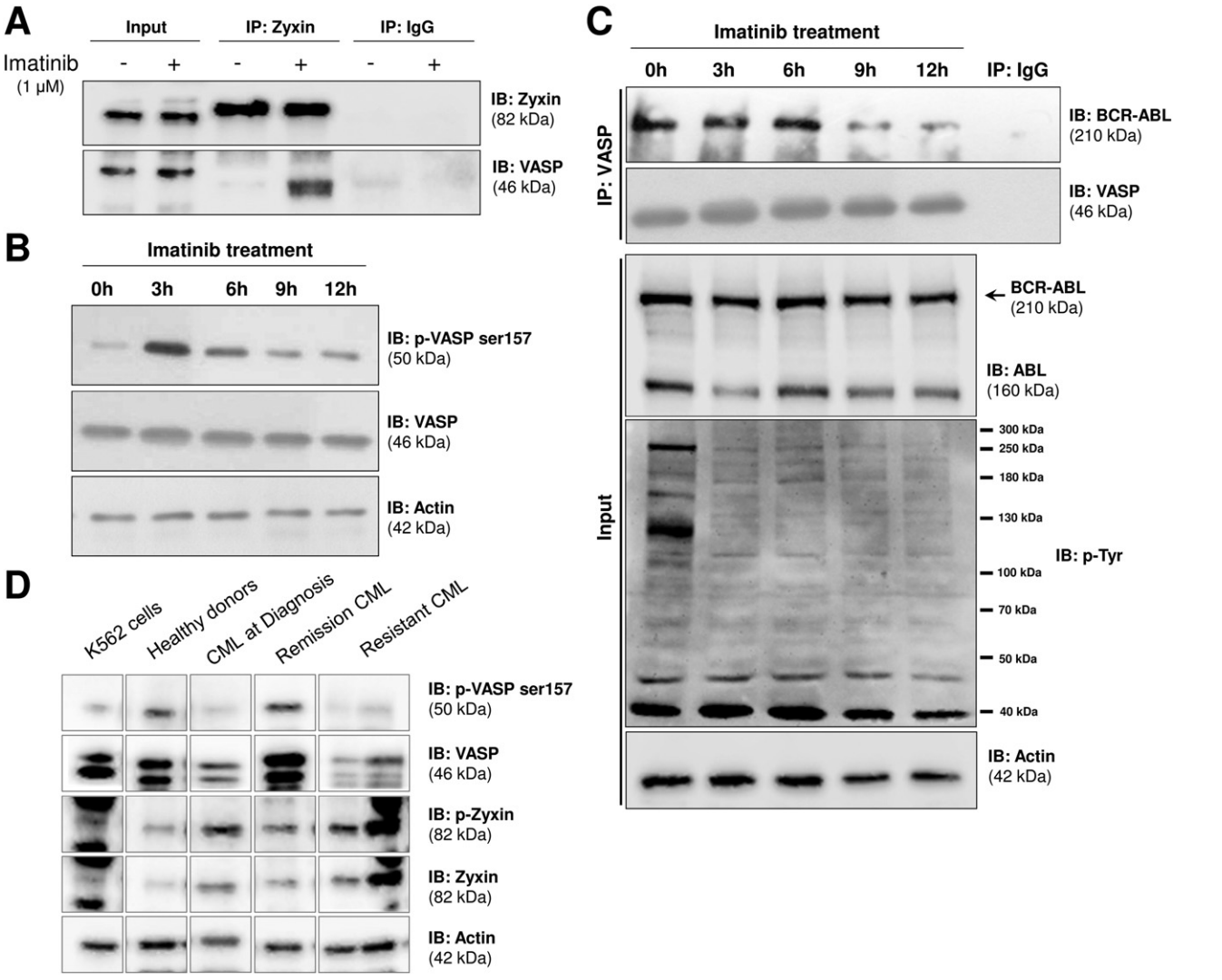


**Fig. 4.** Imatinib treatment enhances VASP and Zyxin interaction, increases VASP phosphorylation at serine 157 and reduces association of VASP and BCR–ABL. (A) The same lysates from K562 cells untreated and treated with Imatinib (1 μM) were immunoprecipitated (IP) with anti-Zyxin antibody and immunoblotted (IB) with anti-Zyxin and anti-VASP antibodies. Total protein extract (input) and isotype IgG antibody were used as controls.(B) Western-blotting using anti-p-VASP ser157, anti-VASP and Actin antibodies in K562 cells treated with Imatinib (1 μM) for 3, 6, 9 and 12 h. (C) Endogenous VASP were co-immunoprecipitated with BCR–ABL. Total extracts from K562 cells treated with Imatinib (1 μM) were submitted to immunoprecipitation with an anti-VASP antibody followed by western blot analysis using anti-ABL and anti-VASP antibody. Total protein extract (input) and isotype IgG antibody were used as controls. (D) Western blotting showed phospho-VASP ser 157, VASP, phospho-Zyxin ser 142 and Zyxin in K562 cells, one healthy donor, one CML patient at diagnosis, one responding and two resistant to Imatinib. Actin staining indicates the amount of protein loaded. All images in Fig. 4E belong to the same western blotting and the membrane was stripped and reprobed with different antibodies. Actin was used as a control to ensuring equal sample loading; the antibodies used for immunoblotting (IB) are indicated.

idea, we observed an increased phosphorylation of VASP on Ser157 status in K562 cells treated with Imatinib, strengthening the idea of phosphorylation at Ser157 in VASP role in CML pathogenesis and in Imatinib treatment.

In Drosophila, Abl regulates subcellular localization of Ena and in the absence of Abl, Ena localizes to ectopic sites [38]. Abl may prevent Ena from binding to partners that localize at cortical sites or may promote Ena binding to proteins that maintain Ena in the cytoplasm [38]. In addition, VASP phosphorylated on Ser157 localizes at focal adhesions and PKA stimulation relocates VASP to focal adhesion [19,39]. PKA is a known target of kinase inhibitors [40]; accordingly our findings indicated that Imatinib, a kinase inhibitor, increased phosphorylation at Ser157 in VASP and disrupted VASP–ABL interaction in BCR–ABL cells. Thus VASP interacted with Zyxin. In CML disease progression, cancerous infiltration of liver, kidney and spleen occurs as a consequence of altered CML cell adhesion to bone marrow stromal cells [28]; the absence of VASP and Zyxin interaction, a key regulator complex of cell–cell adhesion, may contribute to CML defective adhesion.

We demonstrated that VASP affects FAK phosphorylation, pointing towards a possible role for VASP in controlling the migration and adhesion of leukemic cells in CML patients through FAK activity also. In addition, FAK has been shown to play a role as an anti-apoptotic protein [41]. Previous studies have shown that the reduction of BCL-2 or BCL-XL, or overexpression of proapoptotic protein (*e.g.* BIM, BAD) contributes to the better Imatinib response of K562 cells [3,42,43]. Therefore, components that mimic BH3-only protein, which inhibits the anti-apoptotic function of some members of the BCL2 family, are being used in an attempt to overcome resistance to tyrosine kinase inhibitors [3–6]. Our findings that VASP depletion affected the expression of anti-apoptotic proteins are consistent with this idea.

Herein we pointed towards a possible involvement of the absent phosphorylation of VASP at ser157 in CML cells and the lack of VASP and Zyxin interaction in K562 cells into the defective cellular adhesion of CML cells to stromal cells, which implicates in migration and invasion of leukemic cells. Our results suggest a crucial participation of VASP on Imatinib treatment, where phosphorylation at Ser157 in VASP disassociates from ABL and VASP interacts to Zyxin. Future studies targeting VASP and Zyxin in primary CML cells may provide more information about the function of these proteins in the CML phenotype.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.11.008.

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## Competing interests

The authors declare that they have no competing interests.

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